INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS-1 AND -3 AND SEX HORMONE-BINDING GLOBULIN: STUDIES ON REGULATION OF SYNTHESIS *IN VITRO*, AND CLINICAL ASPECTS

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Academic Dissertation

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I Kalme T, Loukovaara M, Koistinen H, Koistinen R, Leinonen P. Comparative studies on the regulation of IGFBP-1 and SHBG production by insulin and insulin-like growth factors in human hepatoma cells. J Ster Biochem Molec Biol 86:197-200, 2003.

II Kalme T, Loukovaara M, Koistinen R, Koistinen H, Angervo M, Leinonen P, Seppälä M. Estradiol increases the production of sex hormone-binding globulin but not insulin-like growth factor binding protein-1 in cultured human hepatoma cells. Fertil Steril 72:325-329, 1999.

III Kalme T, Loukovaara M, Koistinen H, Koistinen R, Seppälä M, Leinonen P. Factors regulating insulin-like growth factor binding protein-3 secretion from human hepatoma (HepG2) cells. J Ster Biochem Molec Biol 78:131-5, 2001.

IV Kalme T, Seppälä M, Qing Q, Koistinen R, Nissinen A, Harrela M, Loukovaara M, Leinonen P, Tuomilehto J. Sex hormone binding globulin and insulin-like growth factor binding protein-1 as indicators of metabolic syndrome, cardiovascular risk and mortality in elderly men. J Clin Endocrinol Metab 90:1550-1556, 2005.

V Loukovaara M, Koistinen R, Kalme T, Kurki T, Leinonen P, Seppälä M. Serum insulin-like growth factor-I and insulin-like growth factor binding protein-3 in premature rupture of membranes. Acta Obstet Gynecol Scand 81:905-908, 2002.

ABBREVIATIONS

ALS	acid-labile subunit
BMI	body mass index
cAMP	cyclic adenosine 3’5’.monophosphate
cDNA	complementary deoxyribonucleic acid
CHD	coronary heart disease
CRP	c-reactive protein
CVD	cardiovascular disease
GH	growth hormone
HDL	high density lipoprotein
IFG	impaired fasting glucose
IFMA	immunofluorometric assay
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IGFBP-rP	IGFBP-related protein
IGF-R	IGF receptor
IGT	impaired glucose tolerance
IL	interleukin
IR	insulin receptor
IVF	*in vitro* fertilization
kDa	kilodalton
LDL	low density lipoprotein
LGA	large for gestational age
NCEP	National Cholesterol Education Program
OGTT	oral glucose tolerance test
PCR	polymerase chain reaction
PROM	premature rupture of membranes
RNA	ribonucleic acid
SGA	small for gestational age
SHBG	sex hormone-binding globulin
TNF	tumor necrosis factor
WHO	World Health Organization

ABSTRACT

Insulin-like growth factors (IGF-I and IGF-II) are potent metabolic and mitogenic proteins with functions in glucose and lipid metabolism as well as cell growth, differentiation and apoptosis. Their activity is controlled by binding proteins (IGFBPs), which also have IGF-independent cell functions. SHBG is another multifunctional protein with direct cell actions as well as binding protein functions.

The aim of the *in vitro* study was to investigate the hepatic regulation of IGFBPs and SHBG using hepatoma cell cultures as experimental models. Insulin and IGFs inhibited IGFBP-1 and SHBG production equipotentially. Estradiol did not affect IGFBP-1 production, but stimulated that of SHBG. Insulin, IGF-I and IGF-II stimulated and cortisol inhibited IGFBP-3 production, whereas sex hormones had no effect at physiological concentrations.

Being liver-derived proteins downregulated by insulin, IGFBP-1 and SHBG could serve as indicators of metabolic syndrome and hyperinsulinemia-related cardiovascular risk. Hence circulating concentrations of SHBG and IGFBP-1 were measured in 335 elderly men. Subjects with metabolic syndrome or abnormal glucose tolerance had low serum SHBG and IGFBP-1 concentrations, but diabetics only low SHBG levels. SHBG was less influenced by BMI than IGFBP-1. Low SHBG levels indicated increased cardiovascular mortality, whereas those of IGFBP-1 had no association with mortality. In women with preterm rupture of membranes and subsequent delivery, serum IGF-I and IGFBP-3 levels did not differ from those in controls.

In conclusion, in *in vitro* conditions, insulin inhibits IGFPB-1 and SHBG production and stimulates IGFPB-3 production in hepatoma cells. Men with low serum SHBG levels had increased cardiovascular mortality, suggesting a possible role of insulin. In women with premature delivery, IGF-1 and the major carrier IGFPB-3 do not play a role in prediction of outcome.

INTRODUCTION

Insulin-like growth factors (IGFs) and the major modulators of their actions, insulin-like growth factor binding proteins (IGFBPs), as well as SHBG, have become the subjects of renewed interest in recent years. IGFBP-1, IGFBP-3 and SHBG are proteins that were long thought to be simple carrier proteins for IGFs and sex hormones, respectively. The new awareness of IGFBPs has arisen as a result of reports demonstrating that they exhibit a multitude of cellular actions that appear to be independent of their IGF-binding functions (Rosenzweig 2004). SHBG has been found to participate in steroid signaling at the cell membrane and local modification of steroid hormone effects (Kahn *et al.* 2002). IGFBPs and SHBG are proteins mainly produced by the liver, but widely expressed in human tissues. They have been found to be multifunctional proteins with roles in glucose metabolism, cell growth and proliferation, the reproductive system, and cardiovascular and cancer risks (Rutanen *et al.* 2000, Kahn *et al.* 2002, Renehan *et al.* 2004, Rosenzweig 2004).

REVIEW OF THE LITERATURE

1. THE INSULIN-LIKE GROWTH FACTOR (IGF) SYSTEM

The IGF family consists of growth factors (IGF-I and IGF-II), specific receptors (IGF-IR, IGF-IIR, IR and hybrid receptors) and binding proteins (IGFBPs) (Jones and Clemmons 1995). The IGFBPs are divided into two groups: high-affinity binding proteins (IGFBP-1 to -6) and low-affinity IGFBP-related proteins (IGFBP-rP1 to -rP4 or IGFBP-7 to -10) (Kim *et al.* 1997).

2. IGF-I AND IGF-II

IGF-I and -II are small single chain peptides that are structurally related to proinsulin. They consist of 70 and 67 amino acids, respectively, with 70% reciprocal homology in humans (Rinderknecht and Humbel 1978a,b). These growth factors, previously designated as somatomedins (Daughaday *et al.* 1972), play many roles in paracrine and endocrine regulation of tissue growth, cell proliferation and differentiation, and apoptosis (Khandwala *et al.* 2000). In mammals they are encoded by large, complex, single copy genes. IGF mRNA or protein can be detected in every tissue examined at some developmental stage (Rechler 1990). IGF peptides occur in large concentrations in the circulation and have systemic, hormonal, and local paracrine effects on cell behavior.

Growth hormone is the main hormonal regulator of circulating IGFs, but other factors such as nutritional status also affect IGF-I concentrations (Jones and Clemmons 1995). One group of regulating factors is the family of IGFBPs, of which IGFBP-3 binds 70–80%, and IGFBP-1 binds 20–30% of IGF-I, leaving only about 10% of IGF-I free. IGF-I receptors are another component of the regulatory system affecting circulating IGF-I. Programmed cell death (apoptosis) can be prevented by IGF-I binding to its receptor (LeRoith *et al.* 1997). Another component of IGF-I regulation is the IGFBP protease system, comprising enzymes that cleave IGFBPs into smaller fragments, thereby altering their abilities to bind IGFs (Wetterau *et al.*

1999). Phosphorylation of IGFBPs also changes their affinity to IGFs (Jones *et al.* 1991, Westwood *et al.* 1997).

IGFs differ functionally from insulin, being subject to long-term nutritional changes and not having acute variation in circulating levels. IGF-I has insulin-like metabolic actions; it plays important roles in regulating glucose metabolism, amino acid and free fatty acid uptake (Boulvare 1992) and the synthesis of various intra- and extracellular proteins such as collagens (Canalis 1980). Serum IGF-I concentrations are reduced in patients with type 1 diabetes and are not normalized by insulin treatment (Holt *et al.* 2003).

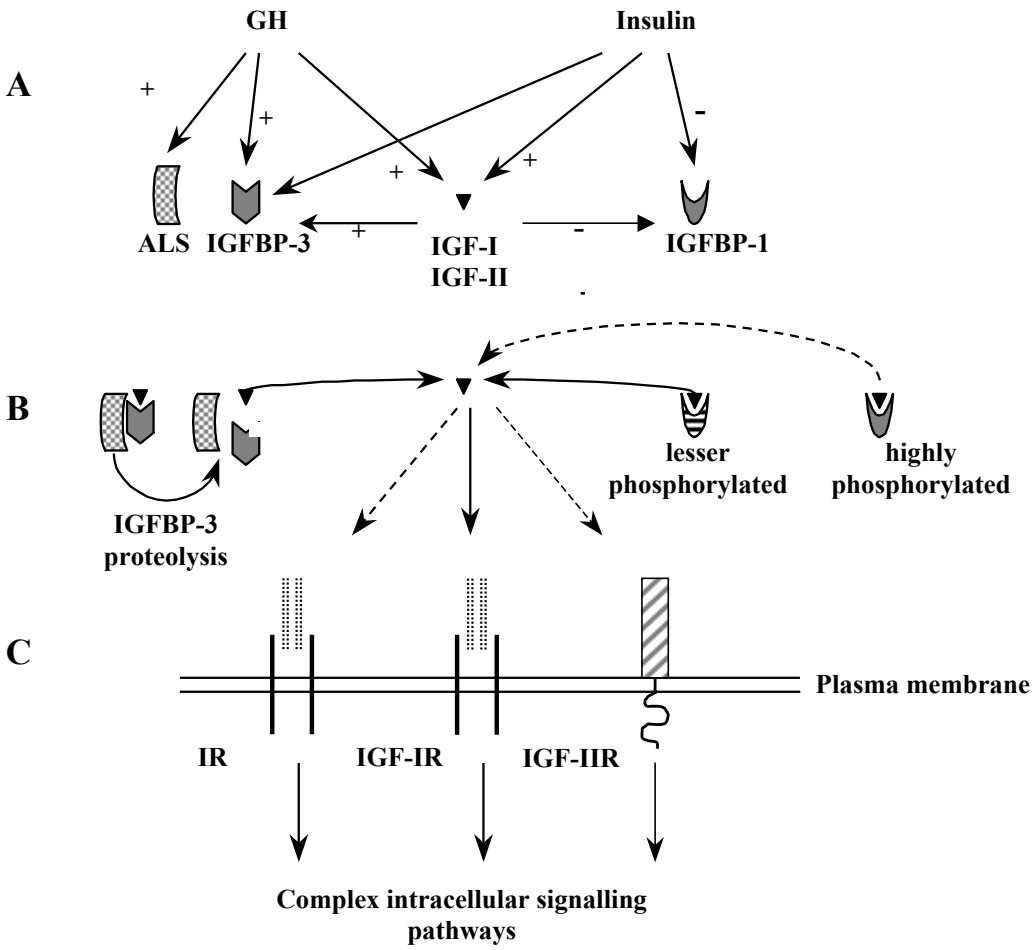
IGF-I and IGF-II stimulate growth by different mechanisms. Studies on knockout mice carrying null mutations of genes encoding IGFs or the IGF-I receptor (IGF IR) revealed that they exhibited severe growth deficiency with underdeveloped bone, muscle and lung tissues (Liu *et al.* 1993). The effects of IGF on the cell cycle consist of promotion of mitotic activity and cell differentiation, as well as inhibition of apoptosis (Jones and Clemmons 1995). High circulating IGF-I concentrations have been found to be associated with an increased risk of prostate, lung, colorectal and premenopausal breast cancer (Reheran *et al.* 2004). Individuals with high serum IGF-II levels also have an elevated risk of developing breast, prostate, colon and lung cancer. IGF-II is commonly expressed by tumor cells and may act as an autocrine growth factor (LeRoith *et al.* 2003). The IGF-I receptor is commonly overexpressed in many cancers, and many recent studies have revealed new signaling pathways emanating from the IGF-I receptor that affect cancer cell proliferation, adhesion, migration and cell death; functions that are critical for cancer cell survival and metastasis (LeRoith *et al.* 2003). Concentrations of IGFs vary greatly between individuals, which might affect the distribution of cancer risk in a population (Pollak 2000).

Figure 1.

A. Main regulatory factors of IGF actions: + = stimulatory effect; - = inhibitory effect

B. Regulation of free IGF availability by IGFBP-1 and IGFBP-3: Lesser-phosphorylated isoforms of IGFBP-1 have reduced affinity for IGF-I and thus are more bound to release. IGF-I and exert lesser inhibitory action. The ternary complex consisting of IGF-I, IGFBP-3and ALS releases free IGF-I after cleavage of IGFBP-3 by specific proteases in the vicinity of IGF receptors.

C. IGF-I and IGF-II bind both to insulin receptor and IGF-IR. The main function of IGF-IIR is to facilitate clearance of IGF-II from circulation.
(Modified from Kajantie 2003 with permission)



3. IGF RECEPTORS

Most of the effects of the IGFs are mediated by binding to IGF-IR. The receptors for insulin and IGF-I are structurally similar, composed of extracellular α subunits that bind the ligands and β subunits that anchor the receptor to the membrane and contain tyrosine kinase activity in their cytoplasmic domains (Jones and Clemmons 1995). IGF-IR binds insulin with approximately 100- to 1000-fold lower affinity compared with IGF-I (Steele-Perkins *et al.* 1988); thus only high concentrations of insulin bind to and activate IGF-IR (Chisalita *et al*. 2004). The affinity of IGF-IR for IGF-II is 2- to 15-fold lower than for IGF-I. Insulin receptors (IRs) have high affinity for insulin and a 100-fold lower affinity for IGF-I (Steele-Perkins *et al.* 1988, Germain *et al.* 1992). The insulin analog glargine has a 10-fold higher affinity for IGF-IR than human insulin (Chisalita *et al.* 2004).

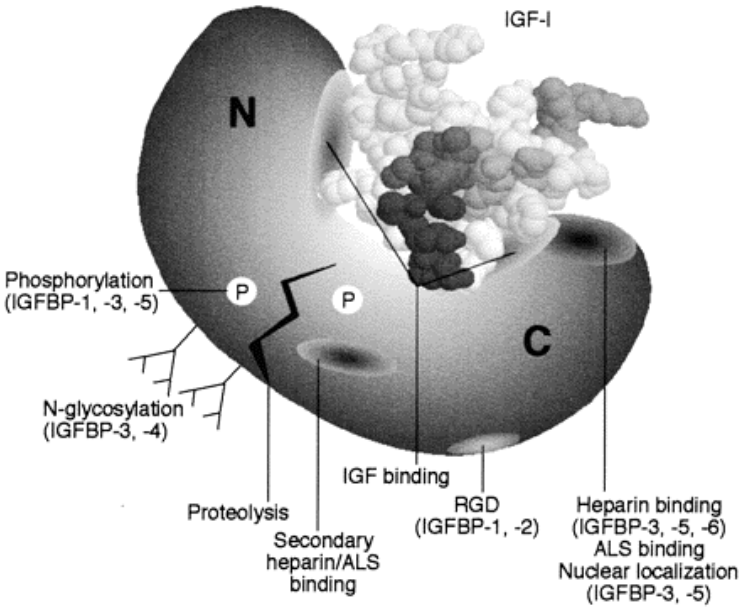
As there is close homology between the IGF-I and insulin receptors, hybrid insulin/IGF receptors may also be formed in tissues coexpressing IR and IGFI-R (Pandini *et al.* 2002). Insulin receptor αβ-half receptors dimerize with IGF-I αβ-half receptors in the presence of ligands (Treadway *et al.* 1992). Hybrids bind both IGF-I and IGF-II with similar affinities as classical IGF-I receptors, but insulin with decreased affinity (Soos *et al.* 1989).

IGF-IIR is structurally unrelated to the other receptors in this family, having a short cytoplasmic tail and no tyrosine kinase activity (LeRoith *et al.* 1995). It is identical to mannose-6-phosphate (M6P) receptor, a protein involved in the intracellular lysosomal targeting of acid hydrolases and other mannosylated proteins (Morgan *et al.* 1987). IGF-IIR is thought to function primarily as a scavenger receptor, regulating the internalization and degradation of extracellular IGF-II, thus regulating circulating IGF-II levels (Khandwala *et al.* 2000). The IGF-II receptor also regulates intracellular trafficking of lysosomal enzymes including cathepsin, which serves as an IGFBP proteolytic enzyme (Khandwala *et al.* 2000).

4. IGF BINDING PROTEINS (IGFBPS)

IGFBPs bind both IGF-I and IGF-II with high affinity. IGFBP-encoding genes share a common structural organization, in which four conserved exons are located within genes ranging from 5 kb (IGFBP-1) to more than 30 kb (IGFBP-2 and IGFBP-5) (Baxter *et al.* 1997). The IGFBPs share distinctive structural and functional characteristics. They contain three structurally distinct domains, each comprising approximately one-third of the molecule (Kiefer *et al.* 1991). All IGFBPs have cysteine-rich amino-terminal (N) and carboxyl-terminal (C) domains and less-conserved central regions (Brinkman *et al.* 1988a). Residues involved in IGF binding occur in both amino- and carboxyl-terminal domains, implying the existence of an IGF-binding pocket involving both domains (Firth and Baxter 2002). Other important subdomains have been identified within the carboxyl-terminal regions of various IGFBPs. Arg-Gly-Asp (RGD) integrin-binding motifs are found in IGFBP-1 and -2. Motifs with heparin-binding activity in IGFBP-3 and -5 are also involved in interaction with the acid-labile subunit (ALS) and other ligands such as PAI-I and transferrin, and with cell and matrix binding and nuclear transport (Firth and Baxter 2002) The nonconserved central domain contains several sites of posttranslational modification: sites of N-linked glycosylation in IGFBP-3 and -4, phosphorylation sites in IGFBP-1, -3 and -5 and proteolytic cleavage sites (Firth and Baxter 2002) (Figure 2).

Figure 2. Generalized diagram of IGFBP structure showing proposed interaction with IGF-I through both N and C domains. Functional domains and sites of posttranslational modification are indicated. (from Firth and Baxter 2002, published by permission)



The actions of IGFBPs can be divided into IGF-dependent and IGF-independent activities (Oh *et al.* 1993a). The IGF-dependent actions include: 1) transport of IGFs in plasma and other biological fluids, 2) increasing the half-life of IGF peptides and 3) modulating the access of IGF ligands to their receptors. IGF-independent actions refer to the ability of IGFBPs to regulate cell growth, migration or metabolism in manners independent of IGF action. IGF-independent actions presumably occur via direct interactions of the IGFBPs with cellular receptors or interacting proteins (Oh *et al.* 1993b).

The six IGFBPs differ in their tissue expression and their regulation from other hormones and growth factors. Their effects are also influenced by posttranslational modifications, such as glycosylation, affecting cell interaction, phosphorylation, affecting IGF-binding affinity and susceptibility to proteases, and proteolysis, affecting both IGF-independent and IGF-dependent actions (Firth and Baxter 2002).

In addition to the six IGFBPs that bind IGFs with high affinity, four IGFBP-related proteins (IGFBP-rPs 1–4) have been identified (Kim *et al.* 1997). IGFBP-rP1 was formerly known as mac25, TAF or PSF; later as IGFBP-7. IGFBP-rPs 2–4 (CTGF, nov, cyr61) belong to the connective tissue growth factor family. They share the cysteine-rich N-terminal region of classical IGFBPs, but not the conserved C-terminal domain required for optimal IGF binding (Baxter *et al.* 1998). Thus IGFBP-rPs bind IGFs with much lower affinity than IGFBPs.

This review of the literature concentrates further on IGFBP-1 and -3, which are probably the most significant circulating IGFBPs (Rajaram *et al.* 1997).

5. IGFBP-1

IGFBP-1, formerly also known as PP12, was the first of the IGFBPs to be purified and to have its cDNA cloned (Brinkman *et al.* 1988a, Julkunen *et al.* 1988). It is an important IGFBP as regards regulation of IGF availability and actions in most human tissues.

5.1 Gene and protein structure

The human IGFBP-1 gene is a single copy gene, located in chromosome 7 (p12-p13) (Brinkman *et al.* 1988a, Alitalo *et al.* 1989). The gene spans 5.2 kb and is divided into 4 exons (Brinkman *et al.* 1988b). The 25 kDa IGFBP-1 protein sequence contains 12 N-terminal and 6 C-terminal cysteine residues, which are also found in other IGFBPs and other mammalian IGFBP-1 sequences. Optimal IGF binding requires both cysteine-rich regions (Brinkman *et al.* 1991). The IGFBP-1 mid-region acts as a specific target for protease activity as well as a hinge defining ligand-binding characteristics (Lee *et al.* 1993).

5.2 Production sites

The IGFBP-1 gene is primarily expressed in the liver (Brinkman *et al.* 1988a, Julkunen *et al.* 1988), but also in decidualized endometrium (Rutanen *et al.* 1985, Julkunen *et al.* 1988), ovarian granulosa cells (Suikkari *et al.* 1989a) and kidney (Chin *et al.* 1992, Suikkari *et al.* 1992). Expression of IGFBP-1 is also found in human coronary arteriectomy specimens (Grant *et al.* 1996).

5.3 Function

Together with the other IGFBPs, IGFBP-1 is an important regulator of local bioactivity of the insulin-like growth factors in various tissues (Shimasaki *et al.* 1991). In contrast to IGFBP-3, IGFBP-1 is for the most part unbound in serum, and it regulates acute changes in serum IGFs (Rajaram *et al.* 1997). It is believed that IGF-I can leave the circulating reservoir only in its free form or when bound to IGFBP-1 or -2 (Juul *et al.* 2002). Two major roles for IGFBP-1 in normal physiology have been suggested: 1) as an endocrine factor, IGFBP-1 regulates the bioavailability of serum IGF-I, thereby modulating IGF-mediated tissue metabolism; 2) as an autocrine/paracrine factor, IGBP-1 plays an important role in the female reproductive system, from ovulation and implantation to successful fetal outcome (Lee *et al.* 1997).

5.4 Phosphorylation

IGFBP-1 exists as five different phosphoisoforms. Phosphorylation of IGFBP-1 is reported to increase the affinity for ligands and thus influence the ability of IGFs to interact with their receptors (Jones *et al.* 1991, Westwood *et al.* 1997). Phosphorylated IGFBP-1 variants have been identified in amniotic fluid, serum and decidua (Koistinen *et al.* 1993a). The variant present in nonpregnant human serum is highly phosphorylated. However, the degree of phosphorylation in maternal plasma varies during pregnancy so that nonphosphorylated and three lesser phosphorylated variants have been observed (Koistinen *et al.* 1993b, Westwood *et al.* 1994) The affinity of the highly phosphorylated form of IGFBP-1 for IGF-I is significantly greater than that of IGFBP-3, the main carrier of IGF in blood (Westwood *et al.* 1997). It appears that normally, IGFBP-1 in the circulation inhibits IGF actions; however, changes in IGFBP-1 phosphorylation status may permit increased IGF bioavailability.

5.5 Regulation

HepG2 cell cultures

The human HepG2 hepatoma cell line has been used to study regulation of IGFBP-1 production *in vitro* (Conover *et al.* 1990). Earlier studies have shown an inhibitory effect of insulin on the production of IGFPB-1 (Conover *et al.* 1990, Lee *et al.* 1993). The effects of IGF-I on IGFBP-1 production have been described as variable, ranging from inhibitory to no effect at all (Conover *et al.* 1990, Singh *et al.* 1990). IGF-II appears to have an inhibitory effect on IGFBP-1 (Lee *et al.* 1993). Glucocorticoids and cAMP stimulate IGFBP-1 transcription (Conover *et al.* 1993). Epidermal growth factor (Angervo *et al.* 1992), tri-iodothyronine (Angervo *et al.* 1993a) and the cytokines TNF-α, IL-1β and IL-6 (Samstein *et al.* 1996, Benbassat *et al.* 1999) increase the synthesis of IGFBP-1 in HepG2 cells (Table 1).

Table 1. Summary of previous *in vitro* studies of IGFBP-1, IGFBP-3 and SHBG production in HepG2 cells. (For references, see text)

	IGFBP-1	SHBG	IGFBP-3
Insulin	↓	↓	ND
IGF-I	↓ / –	↓	ND
IGF-II	↓	ND	ND
GH	ND	ND	↑
estradiol	ND	↑ / –	ND
tamoxifen	ND	↑ / –	ND
testosterone	ND	↑ / ↓	ND
T3	↑	↑ / ↓	ND

ND: not determined

Physiological conditions

IGFBP-1 differs from other IGFBPs, as plasma concentrations show marked diurnal variation, first reported among pregnant women (Rutanen *et al.* 1984). In the nonpregnant state, plasma levels show a difference of over 10-fold between midnight and morning regardless of metabolic and hormonal status (Baxter *et al.* 1987). These changes are obviously associated with nutritional factors, with elevated values during fasting (Busby *et al.* 1988, Hall *et al.* 1988).

The concentration of IGFBP-1 in human fetal serum is 10 times higher than in cord plasma of term infants. The levels continue to decline after birth until a steady state is reached at puberty (Hall *et al.* 1988). Serum IGFBP-l concentrations decline in adolescence (Argente *et al.* 1993) and remain low in adulthood. With advancing age, there may be a slight increase in serum IGFBP-l concentrations as the inverse correlation with insulin levels becomes less pronounced (Rutanen *et al.* 1993).

IGFs and IGFBPs are produced locally by cells of the cardiovascular system, vascular smooth muscle cells and endothelial cells (Delafontaine 1995). They act as growth promoters for arterial cells and mediators in cardiovascular disease (Bayes-Genis *et al.* 2000).

Pathological conditions

Insulin is the primary determinant of IGFBP-1 expression (Suikkari *et al.* 1988, Lee *et al.* 1997). Studies in patients with type 1 diabetes have confirmed that insulin potently suppresses the hepatic output of IGFBP-1 (Brismar *et al.* 1994). IGF-I and IGF-II also have specific inhibitory effects on IGFBP-1 expression. Other factors stimulating IGFBP-1 expression include thyroid hormones (Angervo *et al.* 1993b). Progesterone and other factors that enhance decidualization induce IGFBP-1 production in the endometrium (Gao *et al.* 1999). The relationships between IGFBP-1, type 2 diabetes and cardiovascular risk are reviewed in a separate chapter.

6. IGFBP-3

6.1 Gene and protein structure

The human IGFBP-3 gene is a single copy gene located on chromosome 7. The gene spans 8.9 kilobases and the protein-coding region is divided into four exons while the fifth exon contains a 3'-untranslated region (Cubbage *et al.* 1990). There is close physical linkage between the IGBP-1 and IGBP-3 genes; they are isolated, but are arranged in a tail-to-tail fashion separated by 20 kb of DNA (Ehrenborg *et al.* 1992).

Insulin-like growth factor binding protein-3 is a 40–45 kDa glycoprotein sharing the basic structure of other IGFBPs, with conserved amino- and carboxyl-terminal regions and a unique central region. IGFBP-3 is characterized by its ability to bind to another glycoprotein, the 85 kDa acid-labile subunit (ALS), in the presence of either IGF-I or IGF-II to form a ternary complex of 150 kDa (Baxter *et al.* 1989). The protein-protein and protein-cell interactions of IGFBP-3 are complex and involve distinct domains of the protein. The structural integrity of the IGF-I binding site is disrupted significantly by deletion of either the central or carboxyl-terminal region of IGFBP-3, but more specific mutations of the carboxyl-terminal region can reduce IGF-I binding. The IGF-I and ALS binding sites are functionally distinct (Firth *et al.* 1998).

6.2 Function

IGFBP-3 is the major IGF carrier in human serum. It is synthesized in many tissues, mainly in the liver (Clemmons *et al.* 1991, Donaghy *et al.* 1995). Its serum levels are more than 10-fold higher than those of the other IGFBPs and over 80% of circulating IGF-I is bound to IGFBP-3. IGFBP-3 circulates in a 150 kDa ternary complex together with IGF-I or IGF-II and ALS. This complex extends the half-life of IGF-I in serum to approximately 15 h, compared with 20–30 min for the IGF-IGFBP-1 complex and 10–12 min for free IGF-I (Guler *et al.* 1989). Formation of the ternary complexes restricts the IGFs to the circulation as it prevents IGFs and IGFBP-3 from crossing the capillary barrier (Baxter 1994).

IGFBP-3 has been shown to have direct IGF-independent cellular effects, such as stimulation and inhibition of cell proliferation (Jones and Clemmons 1995), induction of apoptosis (Gill *et al.* 1997, Rajah *et al.* 1997) and nuclear translocation (Jaques *et al.* 1997). It is thought that cell-surface association is needed for many of these functions (Firth *et al.* 1999), but no specific receptor has been identified. However, high IGFBP-3 concentrations are associated with a decreased risk of cancer (Renehan *et al*. 2004).

6.3 Regulation

Specific proteases for IGFBP-3 have been described, including serine proteases, cathepsins and matrix metalloproteinases (Wetterau *et al.* 1999). Proteolysis results in the formation of fragments with 23- to 30-fold decreased affinity for IGFs, thus regulating the bioavailability of IGFs.

Another mechanism of posttranslational modification of IGFBP-3 is glycosylation. The amino acid sequence of IGFBP-3 has three potential N-glycosylation sites in the central region (Firth *et al.* 1999). Human IGFBP-3 is usually found as two differently glycosylated forms of 40–45 kDa (Tressel *et al.* 1991). The state of glycosylation has not been found to affect IGF- or ALS-binding *in vitro* (Conover 1991, Firth *et al.* 1999). While glycosylation does not appear to play a role in IGFBP-3 ligand binding, the level of glycosylation has the potential to modulate the cell-binding activity of IGFBP-3 (Firth *et al.* 1999).

Human hepatoma cell cultures

Serum IGFBP-3 levels are regulated by the GH/IGF-I system so that growth hormone stimulates IGF-I, which in turn directly increases IGFBP-3 secretion (Jørgensen *et al.* 1991). IGFBP-3 cDNA has been cloned from a human liver cDNA library (Wood *et al.* 1988, Spratt *et al.* 1990). However, isolated rat hepatocyte cultures do not release IGFBP-3, and only nonparenchymal rat liver cells have been shown to contain its mRNA (Takenaka *et al.* 1991, Villafuerte *et al.* 1994). Cultures of human liver hepatocytes, lipocytes and Kupfer cells have been demonstrated to contain both IGFBP-3 mRNA and the corresponding immunoreactive protein (Arany *et al.* 1994). The direct effect of insulin on IGFBP-3 production *in vitro* in hepatoma cells has not been studied previously. The effect of IGF-I on IGFBP-3 production has been tested with two hepatoma cell lines, PLC (Scharf *et al.* 1998) and SKHEP (Gucev *et*

al. 1997), as has the effect of IGF-II in PLC cells, but not with the HepG2 cell line, which is known to secrete many proteins of normal liver tissue (Knowles *et al.* 1980) (Table 1).

Physiological conditions

The serum concentrations of immunoreactive IGFBP-3 are low at birth, increase 3-fold in children towards the end of puberty, and then gradually decline from age 18 to 65 (Baxter 1986, Koistinen *et al.* 1994). Circulating levels of IGFBP-3 fluctuate concomitantly with GH levels (Baxter 1986), and GH appears to play a dominant role in the regulation of IGFBP-3 in humans. Other factors influencing IGFBP-3 production are less well known. Unlike IGFBP-1, the circulating concentrations of IGFBP-3 show no diurnal variation (Clemmons *et al.* 1992). A single subcutaneous injection of human recombinant IGF-I has been shown to increase serum IGFBP-1 levels without affecting IGFBP-3 concentrations in normal subjects (Hizuka *et al.* 1993), whereas the administration of IGF-I to patients with non-insulin-dependent diabetes mellitus decreases serum IGFBP-3 levels (Lieberman 1992).

Pathological conditions

Disease states associated with altered serum IGFBP-3 concentrations include acromegaly (elevated levels), growth hormone deficiency and poorly controlled diabetes (subnormal levels) (Baxter 1986, Blum *et al.* 1990, Gargosky 1992). Slightly increased concentrations are seen in renal failure and third-trimester pregnancy (Blum *et al.* 1990, Gargosky 1992). Patients with liver cirrhosis have very low circulating IGFBP-3 levels that correlate with the degree of general liver dysfunction (Møller *et al.* 1995). This is not a result of increased IGFBP-3 proteolysis (Bang *et al.* 1994).

7. SHBG

SHBG is a multifunctional protein that acts in humans to regulate the response to steroids. It is the major binding protein for sex steroids in plasma, thereby determining the availability of free steroids to hormone-responsive tissues (Siiteri *et al.* 1982). It binds testosterone with high affinity and estrogens with lower affinity. Considerable variations in serum SHBG concentrations exist between individuals, depending on nutritional (Anderson *et al.* 1987, Kiddy *et al.* 1989), gender (Vermeulen *et al.* 1969), and hormonal status. SHBG also

functions as part of a steroid-signaling system that is independent of the classical intracellular steroid receptors (Kahn *et al.* 2002).

7.1 Gene and protein structure

The SHBG gene is located in chromosome region 17p13 and comprises at least 8 exons (Bérubé *et al.* 1990). The location is only 30 kb from the p53 tumor suppressor gene, in a region known to undergo allelic deletions and mutations in many tumor types (Cousin *et al.* 2000). The question has been raised of whether genomic changes that alter the SHBG locus might lead to changes causing hormone-dependent cancers (Kahn *et al.* 2002).

Human SHBG is a homodimeric glycoprotein, each monomer consisting of 373 amino acid residues (Walsh *et al.* 1986, Hammond and Bocchinfuso 1996) and containing a steroid-binding site within an amino-terminal laminin G-like (LG) domain (Grishkovskaya *et al.* 2000). Both androgens and estradiol compete for occupancy of this binding site, but appear to enter the site differently and are bound in opposite orientations (Grishkovskaya *et al.* 2000). Occupancy of the human SHBG steroid-binding site by estradiol is also accompanied by a specific conformation of amino acid residues on the surface of the protein that is not seen when the site is occupied by C19 steroids or the synthetic progestin levonorgestrel (Avvakumov *et al.* 2002).

7.2 Function

SHBG has two apparently disparate functions. It is the major regulator of free estrogens and androgens in plasma (Rosner *et al.* 1996) and it is an initiating component of a signaling system for estrogens and androgens at the cell membrane that functions via a G protein (Nakhla *et al.* 1999) and cAMP (Fortunati *et al.* 1999).

SHBG functions as a modulator of androgen delivery to tissues. The unbound fraction of testosterone is bioavailable (Vermeulen *et al.* 1971). In the blood, changes in SHBG concentrations influence the distribution of sex hormones between the free and protein-bound fractions, of which only the free fraction is available for target cell uptake. In women, low concentrations of SHBG have been observed in conditions associated with excessive androgen action, such as hirsutism and acne (Anderson *et al.* 1974), and endometrial cancer

(Lukanova *et al.* 2004). In addition to natural steroids, SHBG binds several synthetic steroids, including the progestins used in contraceptives and hormonal replacement therapies (El Makhzangy *et al.* 1979).

A specific, high-affinity SHBG receptor (R SHBG) has been found in cell membranes of the endometrium, prostate, placenta, breast, liver and epididymis. Unoccupied SHBG binds to the receptors on the cell membrane, followed by binding of steroid to the complex. Activation of R SHBG induces the synthesis of cAMP (Kahn *et al.*2002).

7.3 Production sites

Serum SHBG is mainly produced by the liver. Cultured hepatoma cells secrete SHBG and cDNAs coding for SHBG are found in human liver cDNA libraries (Khan *et al.* 1981, Hammond *et al.* 1987). It is becoming increasingly obvious that expression of the SHBG gene is far more complex than originally assumed and it is responsible for several related gene products in a wide range of tissues, including the breast, prostate, brain, placenta and endometrium (Hammond and Bocchinfuso 1996, Kahn *et al.* 2002). The biological significance of these gene products remains to be determined. Local production of SHBG in breast and prostate cells is probably independent of serum SHBG (Kahn *et al.* 2002). R SHBG signaling has been found to affect growth in human breast carcinoma and human prostate cell lines (Fortunati *et al*. 1996, Nakhla and Rosner 1996). It has been hypothesized that perturbations of SHBG expression in cancer cells might contribute to the malignant phenotype (Kahn *et al*. 2002).

7.4 Regulation

Human hepatoma cell cultures

Inhibitory effects of insulin and IGF-I on the production of SHBG in HepG2 cells have been shown in previous studies (Plymate *et al.* 1988, Singh *et al.* 1990, Crave *et al.* 1995), even in the intracellular compartment (Loukovaara *et al.* 1995). The effect of IGF-II on SHBG production in liver or hepatoma cells is not known. Thyroid hormones increase the secretion of SHBG *in vitro* (Mercier-Bodard *et al.* 1989). Androgens have been found to increase SHBG production in most studies (Lee *et al.* 1987, Mercier-Bodard *et al.* 1987, Plymate *et al.*

1988, 1990). Estrogens and tamoxifen also increase SHBG production (Plymate *et al.* 1988, 1990), but progestin has no effect (Mercier-Bodard *et al.* 1986, Lee *et al.* 1987) (Table 1).

Physiological conditions

Insulin is thought to be the most important regulator of SHBG metabolism *in vivo* (Nestler *et al.* 1993). Other regulators of SHBG production are sex steroids and thyroid hormones.

In fetal plasma, the SHBG level is low but it rises in newborns (August *et al.* 1969). The concentration is stable thereafter, until it decreases during prepuberty in both sexes (Horst *et al.* 1977). After puberty mean values clearly differ between the sexes, with higher values in women than in men (Vermeulen *et al.* 1969). Serum SHBG concentrations rise with age (Maruyama *et al.* 1984). In men, approximately 44% of circulating testosterone is bound to SHBG. In women, a substantial proportion of estradiol, 37–88% depending on the phase of the menstrual cycle, circulates in complex with SHBG (Dunn *et al.* 1981). The menstrual cycle has not been found to affect SHBG levels (Pearlman *et al.* 1967). During pregnancy SHBG levels rise and reach a plateau at 25–30 weeks, being about ten-fold higher than in nonpregnant women (Pearlman *et al.* 1967). At menopause, the decrease in estrogen levels is not reflected in changes in SHBG levels in plasma (Longcope *et al.* 1987). Dietary factors can affect the SHBG level. Fasting increases it, probably by lowering insulin levels (Kiddy *et al.* 1989). Levels of SHBG are also higher during a high carbohydrate diet than a high protein diet (Anderson *et al.* 1987).

Pathological conditions

Obesity is the most common condition affecting circulating SHBG levels, and in obese individuals SHBG concentrations are low in both sexes (Glass *et al.* 1977). This suppression appears to be a result of the effects of insulin on SHBG production (Peiris *et al.* 1989). Abnormally low serum SHBG levels are frequently found in women with polycystic ovarian syndrome (PCOS), and they contribute to hyperandrogenic symptoms such as hirsutism and acne (Anderson 1974). Serum SHBG levels are also reduced in patients with type 2 diabetes, and CHD (Lapidus *et al.* 1986, Lindstedt *et al.* 1991).

Hypothyreosis decreases and hyperthyreosis increases serum SHBG levels (Olivo *et al.* 1970). These changes probably result from direct effects of thyroid hormones on hepatic SHBG production. In cases of male hypogonadism such as Klinefelter's syndrome, SHBG levels are usually within the female range (DeMoor *et al.* 1969). In some conditions, such as Cushing's syndrome and acromegaly, low SHBG levels are most likely caused by underlying disturbances in glucose metabolism, as the majority of these patients have impaired glucose tolerance or even diabetes (Daughaday 1995).

In the majority of studies on SHBG levels and prostate cancer no differences have been found between cases and controls (Shaneyfelt *et al.* 2000), but in one prospective study with multivariate adjusted data men with the highest circulating SHBG levels were less likely to develop prostate cancer (Gann *et al.* 1996). Local SHBG production might be more important as regards cancer development (Kahn *et al.* 2002).

8. Regulation of IGFBP-1 and SHBG by insulin and IGFs

Since portal hyperinsulinemia is a key feature of metabolic syndrome (Björntorp *et al.* 1988), the serum IGFBP-1 concentration might serve as a marker of hyperinsulinemia and insulin resistance by indicating insulin bioactivity in the portal vein (Yki-Järvinen *et al.* 1995). An easily measurable marker is needed, as the insulin clamp technique, the current gold standard for assaying insulin sensitivity (DeFronzo *et al.* 1979), is an invasive and technically demanding procedure not suited for large populations. The results of clinical studies indicate that IGFBP-1 concentrations correlate with cardiovascular risk factors (Harrela *et al.* 2000). Clinical studies have also shown an association between SHBG and hyperinsulinemic insulin resistance (Nestler *et al.* 1993).

Earlier studies on HepG2 cells have shown an inhibitory effect of insulin on the production of both IGFBP-1 and SHBG (Plymate *et al.* 1988, Conover *et al.* 1990, Singh *et al.* 1990 Lee *et al.* 1993, Crave *et al.* 1995), while the effects of IGF-I have been variable, ranging from inhibitory to no effect at all (Singh *et al.* 1990, Crave *et al.* 1995, Loukovaara *et al.* 1995).

The effect of IGF-II is unknown. The relative potencies of insulin and insulin-like growth factors in regulation of the production of IGFBP-1 and SHBG by the liver is still unresolved.

9. IGFBP-1 AND ESTROGENS

9.1 Ovarian stimulation

The ovary is a site of IGF, IGF-IR and IGFBP expression and action. IGFs stimulate ovarian cellular mitosis and steroidogenesis, and IGFBPs are inhibitory to these processes (Mason *et al.* 1992, Giudice *et al.* 1993). IGFBP-1 mRNA has been shown to be present in granulosa cells, notably in the dominant follicle (Koistinen *et al.* 1990, El-Roeiy *et al.* 1994). It is also increased in luteinizing follicles (Thierry van Dessel *et al.* 1996) and in follicles containing mature oocytes (Kawano *et al.* 1997). This suggests a role for IGFBP-1 in development of the dominant follicle and in corpus luteum regulation and thus a possible use as a marker of mature oocytes (Kawano *et al.* 1997, Fried *et al.* 2003).

Both IGF-I and FSH stimulate estradiol and reduce IGFBP-1 production in cultured granulosa cells (Dor *et al.* 1992, Mason *et al.* 1993). *In vivo*, no significant variation in circulating IGFBP-1 concentrations has been observed during the normal menstrual cycle (Suikkari *et al.* 1987, Pekonen *et al.* 1992, van Dessel *et al.* 1996). Ovarian stimulation is followed by rising estrogen and IGFBP-1 levels, both in follicular fluid and serum (Seppälä *et al.* 1984, 1988, Martikainen *et al.* 1992). However, whether the rise in serum levels of IGFBP-1 is caused by stimulation of production in the liver or ovary, or both, has not been determined. Normally, circulating IGFBP-1 is derived mainly from the liver, but in controlled ovarian hyperstimulation the contribution from hyperstimulated preovulatory follicles may be important (Martikainen *et al.* 1991, 1992, Pekonen *et al.* 1992). A recent finding of approximately ten-fold higher levels of IGFBP-1 in follicular fluid during superovulation treatment as compared with serum levels supports this idea (Fried *et al.* 2003). If also GH is given during superovulation treatment, serum IGFBP-1 values decrease significantly, compared with the situation in women receiving only gonadotropins, while IGF-I and insulin values remain unchanged (Tapanainen *et al.* 1991).

9.2. Hormone treatment

Changes in serum IGF-I and IGFBP-1 levels are also found during hormone therapy (HT). In particular, oral estrogen therapy (ET) is associated with a decrease in serum levels of IGF-I and an increase in those of IGFBP-1 (Weissberger *et al.* 1991, Kelly *et al.* 1993, Campagnoli *et al.* 1995, Helle *et al.* 1996, Paassilta *et al.* 2000). During transdermal ET, unaffected IGF-I levels have been reported (Weissberger *et al.* 1991, Bellantoni *et al.* 1996, Paassilta *et al.* 2000). IGF-I levels drop only when exceptionally high doses of transdermal estrogen have been administered (Friend *et al.* 1996). Levels of IGFBP-1 also remain unchanged during transdermal ET. The estrogen antagonist tamoxifen has similar effects on the IGF system as estrogen *in vivo* (Lønning *et al.* 1992, Friedl *et al.* 1993, Decensi *et al.* 1999).

Oral ET exerts its effects on serum IGF-I via a hepatic first pass effect. IGF-I is part of the hypothalamic-pituitary-hepatic axis complex involving GH-releasing and GH-inhibiting hormones (Paassilta *et al.* 2000). Thus, changes in plasma levels of IGF-I could reflect an indirect effect of estrogen, the primary effect being extrahepatic. Animal studies have suggested that the primary target of estrogen action could be the pituitary, and that the IGF-I-lowering effect of estrogen takes place indirectly (Krattenmacher *et al.* 1994). In addition, oral therapy with conjugated estrogen has been reported to increase GH levels in postmenopausal women (Bellantoni *et al.* 1996). During transdermal ET, however, estradiol is delivered directly into the peripheral circulation. Therefore, hepatic exposure to estradiol might be of importance in inducing the changes in the IGF system. It has been proposed that the effects of oral estrogen on the IGF system are mediated, in whole or in part, by down-regulation of growth hormone signaling in hepatocytes (McCarty 2003).

10. METABOLIC SYNDROME, DIABETES RISK AND MORTALITY

Metabolic syndrome involves disturbances in glucose metabolism and insulin secretion, an overweight condition, abdominal fat distribution, dyslipidemia and hypertension. It has a close association with subsequent development of type 2 diabetes and cardiovascular disease (Reaven *et al.* 1988). The underlying abnormality is considered to be insulin resistance. The pathogenesis of metabolic syndrome is not clear, but dietary factors and physical activity

clearly interact in development of the syndrome, together with as yet unknown genetic factors (Liese *et al.* 1998). The diagnosis of metabolic syndrome requires multiple laboratory tests. As the syndrome is becoming increasingly common in the Western world, practical tools for mass screening for it should be helpful.

Formerly, definitions of this syndrome have varied widely and the names metabolic syndrome and insulin resistance syndrome have both been used. The World Health Organization (WHO) and the National Cholesterol Education Program (NCEP) Expert Panel have both published recent definitions (Table 2) (Alberti *et al.* 1998, Balkau *et al.* 1999).

Table 2: WHO and NCEP definitions of the metabolic syndrome in men

WHO Definition	**NCEP Definition**
Hyperinsulinemia (upper quartile of the nondiabetic population) or fasting plasma glucose ≥ 6.1 nmol/l AND	At least 3 of the following:
At least 2 of the following	Fasting plasma glucose ≥ 6.1 nmol/l
Abdominal obesity:	Abdominal obesity:
-Definition 1: Waist hip ratio >90 or BMI = 30	-Definition 1: waist girth > 102 cm
-Definition 2: Waist girth > 94 cm	- Definition 2: Waist girth > 94 cm
Dyslipidemia (serum triglycerides ≥1.7 mmol/l or HDL cholesterol <0.9 mmol/l)	Serum triglycerides ≥1.7 mmol/l Serum HDL cholesterol <0.9 mmol/l
Blood pressure ≥ 140/90 mmHg or medication	Blood pressure ≥ 130/85 mmHg or medication

Recent studies have shown that even subjects with metabolic syndrome but without diabetes have 2–3 fold CVD mortality (Malik *et al.* 2004). Those with diabetes had a 2-fold increase even in overall mortality. Even one or two metabolic syndrome features confer an increased risk of CHD and CVD mortality (Malik *et al.* 2004).

11. IGFBP-1 AND SHBG IN METABOLIC SYNDROME AND DIABETES

The relationship between androgen homeostasis, insulin sensitivity and cardiovascular risk in men is complex. Low levels of SHBG, and free or total testosterone in men have been associated with type 2 diabetes, visceral obesity, insulin resistance or hyperinsulinemia, dyslipidemia and metabolic syndrome (Haffner *et al.* 1996, Laaksonen *et al.* 2003). Testosterone and insulin concentrations are inversely associated in healthy men (Simon *et al.* 1992). Testosterone may have a central role in the pathogenesis of metabolic syndrome or type 2 diabetes, as it increases skeletal muscle tissue and decreases abdominal obesity and nonesterified fatty acids, consequently improving insulin sensitivity (Marin *et al.* 1992).

In contrast to total testosterone, calculated free testosterone levels have not been found to predict the development of metabolic syndrome or diabetes in previous studies (Stellato *et al.* 2000, Laaksonen *et al.* 2004). Because the level of free testosterone is related to that of SHBG, it may be that SHBG is more important than testosterone itself as a risk marker or as a contributor to the development of metabolic syndrome or diabetes (Laaksonen *et al.* 2004). Weight loss and successful weight maintenance increase free and total testosterone and SHBG in men with general obesity or abdominal obesity and metabolic syndrome (Kaukua *et al.* 2003, Niskanen *et al.* 2004).

In relatively small trials androgen treatment has improved insulin sensitivity in middle-aged abdominally obese men (Marin *et al.* 1992, 1995), but the findings have not been consistent (Liu *et al.* 2003). The results of some studies suggest that exogenous administration of androgenic steroids to men or women results in insulin resistance (Cohen *et al.* 1987). In other studies administration of testosterone has improved insulin sensitivity, probably through improvements in body composition and reduction in circulating nonesterified fatty acids (Marin *et al.* 1992, Marin 1995). A window for optimal insulin sensitivity has been suggested, as both elevated and low testosterone concentrations can reduce body sensitivity to insulin (Björntorp 1991). The results of most studies suggest a less atherogenic lipid profile with increasing endogenous total testosterone (Barrett-Connor 1995). Testosterone could be an independent risk factor for cardiovascular diseases or its effects may be mediated via other risk factors such as insulin levels.

Portal hyperinsulinemia is a key feature of metabolic syndrome (Björntorp 1988). SHBG and IGFBP-1 have been suggested as potential indicators of metabolic syndrome and hyperinsulinemia-related cardiovascular risk (Nestler *et al.* 1993). An association between low SHBG levels and the development of type 2 diabetes has also been reported (Lindstedt *et al.* 1991, Birkeland *et al.* 1993, Haffner *et al.* 1993, 1996, Gyllenborg *et al.* 2001).

In insulin clamp studies, the relative insulin-induced decline of IGFBP-1 serum concentrations is 18 times greater than that of SHBG (Ebeling *et al.* 1995). Therefore, IGFBP-1 might correlate better than SHBG with hyperinsulinemia and its related cardiovascular risk factors. Moreover, IGFBP-1 might be devoid of the effects of confounding factors, such as estrogens and testosterone. Estrogen increases SHBG concentrations whereas testosterone decreases both SHBG levels and insulin sensitivity (Björntorp *et al.* 1991).

12. THE IGF SYSTEM IN PREGNANCY

Both maternal and fetal IGFs and IGFBPs play important roles in fetal growth and development (D'Ercole 1992, Chard *et al.* 1994, Tazuke *et al.* 1996). IGFBP-1 is synthesized in large amounts by the secretory endometrium and decidua of early pregnancy. Fetal serum IGFBP-1 is produced mainly in the liver, but also in some other tissues, including the fetal pancreas (Hill *et al.* 1989). In fetal growth disorders changes in the IGF system can be found, IGF-I and IGFBP-1 concentrations being decreased in cord sera from small-for-gestational age (SGA) newborns and increased in large-for-gestational age (LGA) newborns (Verhaeghe *et al.* 1993, Giudice *et al.* 1995). Some investigators have also reported low IGF-II levels in small fetuses (Verhaeghe *et al.* 1993, Giudice *et al.* 1995), while others have shown no such association (Lassarre *et al.* 1991). Low IGFBP-3 and increased IGFBP-1 levels have also been found in cord blood of SGA infants (Giudice *et al.* 1995).

Several changes occur in the IGF system during pregnancy. Maternal serum concentrations of IGF-I rise progressively throughout pregnancy (Wilson 1982, Hall *et al.* 1984, Wang *et al.* 1991), and its levels are determined by the mean concentration of GH derived from the placenta. Levels of IGFBP-1 and IGFBP-3 in maternal plasma also rise with gestational age (Drop *et al.* 1984, Langford 1995). Decidual tissues are the main source of IGFBP-1 in

maternal serum (Rutanen *et al.* 1985). As in the decidua (Koistinen *et al.* 1993b), the phosphorylation state of IGFBP-1 in maternal plasma is altered during pregnancy, as a nonphosphorylated and three less phosphorylated forms appear in the plasma (Westwood *et al.* 1994). Levels of IGFBP-1 are often elevated in preeclampsia (Iino *et al.* 1986), while those of IGFBP-3 remain unchanged (Varma *et al.* 1993).

Maternal levels of circulating IGFBP-3 have been shown to be increased in the third trimester of pregnancy compared with nonpregnant values (Baxter *et al.* 1986). The presence of a specific protease acting on IGFBP-3 was first suspected in 1990 (Fielder *et al.* 1990). Proteolysis of IGFBP-3 has been demonstrated in a variety of conditions. It has been suggested that proteolytic activity increases in the order: acromegalic patients, normal subjects, GH-deficient patients and pregnant women (Lassarre *et al.* 1991). Proteolytic activity is also observed during severe illness (Davies *et al.* 1991) and after surgery (Davenport *et al.* 1992). Proteolysis of IGFBP-3 in maternal serum may provide a mechanism to increase IGF availability required for fetal and placental growth (Rutanen *et al.* 2000).

13. THE IGF SYSTEM IN DELIVERY AND PREMATURE RUPTURE OF FETAL MEMBRANES

The IGF system plays an important role in the onset of labor. *In vitro*, IGF-I decreases the release of placental prostaglandin F2 and thromboxane, both with vasoconstricting properties. This is one of the mechanisms by which IGF-I is believed to increase placental blood flow and maintain pregnancy (Siler-Khodr *et al.* 1993). Concordant with this, serum IGF-I levels are low during delivery in the third trimester, compared with pregnancies without regular uterine contractions (Wang *et al.* 1993). In term and preterm deliveries, the endocrinological changes have been found to be similar, i.e. the cascade leading to preterm birth represents a normal signal with inappropriate timing rather than an abnormal signal (Steer 1990). Serum levels of IGF-I are also similar during term and preterm parturition (Wang *et al.* 1993).

Increased IGFBP-1 levels have been observed during labor as compared with samples from infants delivered by cesarean section before the onset of labor, in cord blood (Hills *et al.* 1994) and maternal serum (Wang *et al.* 1995). Maternal IGFBP-1 levels have been reported to

be inversely related to those of insulin (Wang *et al.* 1995). The reason for IGFBP-1 concentration changes during labor could be acute stress, regulated by insulin (Lee *et al.* 1997).

In spite of increasing knowledge of the etiology and associated changes behind PROM and subsequent preterm delivery, they remain important causes of perinatal mortality and morbidity. Preterm delivery is preceded by premature rupture of membranes (PROM) in about one-third of the cases (Arias *et al.* 1982). Levels of IGF or IGFBP in women with PROM and preterm delivery, before regular uterine contractions appear, have not been studied previously. Premature rupture of membranes invariably leads to preterm delivery sooner or later (Cox 1988) and is associated with chorioamnionitis in up to 70% of cases (Naeye 1980). The role of infection is gaining importance in the etiology of preterm birth. Up to 80% of women who deliver before 30 weeks of gestation have evidence of bacterial infection in the amniotic fluid and/or membranes, compared with only 30% at 37 weeks of gestation (Goldenberg *et al.* 2000). Infections have been found to change IGF-I and IGFBP-3 levels in serum. The levels are low in generalized infection, as shown in children with perinatal human immunodeficiency virus infection and serious disease (de Martino 2000). Low IGFBP-3 levels have also been found in other severely ill patients (Davies 1991) and after surgery (Davenport 1992).

AIMS OF THE STUDY

Circulating IGFBPs and SHBG are important regulators of human glucose and sex hormone metabolism, as well as female reproductive functions. This study was designed to address different aspects of regulation of IGFBP and SHBG production as well as the clinical situations associated with metabolic syndrome and preterm delivery. The specific aims were:

1. To compare the effects of insulin and IGFs on IGFBP-1 and SHBG production in human hepatoma cells.
2. To study IGFBP-1 responses to estradiol in human hepatoma cells using SHBG as a control, and thus to investigate the possible source of elevation of serum IGFBP-1 levels caused by high estradiol values during hormone treatments.
3. To study *in vitro* the effects of insulin, IGF-I, IGF-II, sex hormones and cortisol on IGFBP-3, the GH-regulated major modulator of IGF action in the circulation.
4. To study and compare serum SHBG and IGFBP-1 levels as potential indicators of glucose intolerance, metabolic syndrome, diabetes, cardiovascular risk and CVD mortality in elderly men.
5. To evaluate whether maternal circulating levels of IGF-I and IGFBP-3 are affected in PROM or preterm delivery.

MATERIALS AND METHODS

1. *IN VITRO* STUDIES (I, II, III)

1.1 Cell cultures

Human hepatoma cells (HepG2 cells) were grown at 37 °C in 92% air/8% CO_2, in Dulbecco's Modified Eagle's Medium without phenol red, supplemented (10%) with heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM nonessential amino acids, penicillin (100 U/mL) and streptomycin (100 μg/mL). The HepG2 cells were grown to subconfluence for three days. The subconfluent cell layers were washed twice with phosphate-buffered saline, after which the cells were cultivated for one day under serum-free conditions. The medium was then replaced with fresh serum-free medium, and the cells were cultivated in the presence or absence of hormones for another two days for IGFBP-1, IGFBP-3 and SHBG protein measurements, or for six hours for RNA experiments. For protein assays each experiment was performed three times with six observations for each hormone concentration. At the end of the incubation, the media were removed and stored at -20 °C until assayed. Cell viability was determined by means of trypan blue uptake. The cells for cellular IGFBP-1, SHBG and DNA measurements (I) were washed with PBS, harvested mechanically in 1.5 mL PBS, and stored at -20 °C until being homogenized and assayed. Homogenization was carried out by sonication. The cells for RNA extraction (II, III) were used immediately.

1.2 Assays

IGFBP-1 measurement (I, II, IV)

Concentrations of IGFBP-1 in homogenized HepG2 cells (I), culture media (I, II) and serum (IV) were determined using a specific immunofluorometric assay (IFMA) performed essentially as described previously (Koistinen *et al.* 1996), using two monoclonal antibodies, F34-15C9 and F36-9G3. Briefly, the first antibody was bound to polystyrene microtiter wells and the second antibody was labeled with europium chelate.

The sensitivity of the assay was 0.1 μg/L, intra-assay variation was 3–11%, interassay variation was 4–10% and the linear measuring range was 0.1–100 μg/L.

IGFBP-3 measurement (III, V)

Concentrations of IGFBP-3 in culture media (III) and maternal serum (V) were determined by a highly specific and sensitive immunofluorometric assay described previously (Koistinen *et al.* 1994). Briefly, monoclonal antibodies (Mabs) were generated against recombinant IGFBP-$3^{E.Coli}$. The assay employed Mab F42-1B6 as the solid phase antibody and Mab F41-5C11 as europium-labeled tracer. The intra-assay variation was 3.6–6.2% and the interassay variation 5.4–11%. There are no cross-reactions with other human IGFBPs or IGFs in this assay (Koistinen *et al.* 1994).

SHBG measurement (I, II, IV)

A commercial immunofluorometric assay (Delfia SHBG Assay, Wallac Oy, Turku, Finland) was used, based on the direct sandwich technique in which polyclonal rabbit anti-SHBG antibodies and monoclonal mouse anti-SHBG antibodies are employed. The sensitivity of the assay was 0.01 μg/dL (0.5 nmol/L), intra- and interassay coefficients were 7.8% and 9.1% respectively, and the measuring range was 0.01–5 μg/dL (0.5–200 nmol/L).

IGF-I measurement (V)

Serum concentrations of IGF-I were measured by commercial enzyme-linked immunosorbent assay, following the instructions of the manufacturer (Diagnostic Systems Laboratories, Webster, TX, USA). The intra- and interassay coefficients of variation were 4.5–7.1% and 4.8–8.8%, respectively.

Isolation of total RNA and Northern blotting analysis (I, III)

Total hepatocyte RNA was extracted by treating the cells with 4 M guanidinium isothiocyanate, followed by acid-guanidinium-thiocyanate-phenol-chloroform extraction (Chomczynski *et al.* 1987). RNA was quantified by measuring ultraviolet absorbance at 260 nm.

Total RNA (20 µg) was subjected to electrophoresis and transferred to nylon membrane. For IGFBP-1, the entire 1.5-kb-long human IGFBP-1 cDNA (Villafuerte *et al.* 1994) was used as a probe. The 679-bp-long IGFBP-3 cDNA was cloned from decidua and the 402-bp-long SHBG cDNA from HepG2 cells by RT-PCR. Labeling of EcoRI fragments was performed by means of the rediprime DNA-labeling system. The filter was hybridized overnight at 68 °C using Express Hyb hybridization solution and ^{32}P-labeled IGFBP-1, IGFBP-3 or SHBG cDNA. For autoradiography, the filter was exposed to Hyperfilm-MP film for 12 h. Ribosomal RNA was used to quantify the amount and to control the quality of loaded RNA. The amounts of IGFBP-3 and ribosomal RNA were estimated from autoradiographs and gels, respectively, using a UV transilluminator with and without a UV-to-white converter, respectively, and the GeneTools program.

DNA measurement (II)

The amount of cellular DNA was determined by means of a slight modification of the procedure described by Sorger and Germinario (1983), based on measurement of the relative fluorescence of a DNA-4',6'-diamidino-2-phenylindole complex.

1.3 Statistical methods

The results were expressed as a percentage of the control value in each case, and given as the mean ± SEM of six culture wells for each dose and hormone for protein analysis. The differences in levels between cultures with and without the hormones were evaluated by factorial analysis of variance (I, III) or the Mann–Whitney *U* test (II). A value of $p < 0.05$ was considered statistically significant.

2. CLINICAL STUDIES (IV, V)

2.1 Study Subjects

Study IV

The study subjects came from the Seven Countries Study, initiated as a cardiovascular risk survey among 16 cohorts of men in seven countries in 1959 (Keys *et al.* 1966). The original cohorts of the Finnish part of the study consisted of men born between 1900 and 1919 in two geographically defined rural areas in eastern (n = 823) and south-western (n = 888) Finland (Nissinen *et al.* 1993). Of the original cohort, 524 were alive on the 1st of January 1989, 413 participated in the 30-year examination, and 335 men formed the study group for the present analysis. Reasons for non-participation of these 70- to 89-year-old men included long distance travel, poor health, inadequate fasting and unwillingness to participate. Prevalent diseases in the study population are given in Table 3. All the subjects had given informed consent, and the Ethics Committee of the National Public Health Institute approved the study design.

Table 3. Prevalent diseases in study population (IV)

	n	%
Metabolic syndrome	94	28
Impaired fasting glucose (IFG)	20	6
Impaired glucose tolerance (IGT)	37	11
IFG and IGT	17	5
Diabetes	109	32
Cardiovascular disease (CVD)	184	54

Study V

Thirty-two women were referred to Helsinki University Central Hospital because of premature rupture of the membranes (PROM), defined as an identifiable leakage of amniotic fluid before the 37th gestational week. General characteristics of the study population are

given in Table 4. Women with regular uterine contractions were excluded. All the women except one received a 3-day course of prophylactic cefuroxime. Betamethasone, 12 mg, was given twice, with a 12-hour interval, at < 32 gestational weeks. Twenty-seven healthy pregnant women from antenatal clinics served as gestational age-matched controls. The study was carried out with the approval of the Institutional Review Board of the Department of Obstetrics and Gynecology, Helsinki University Central Hospital, and all the study subjects gave informed consent.

Table 4. Characteristics of the control subjects and PROM patients (V)

	Control subjects (n = 27)	PROM patients (n = 32)
Age (years) (mean ± SEM)	30 ± 1	32 ± 1
Primiparae (%)	63	37
Gestational age at PROM (weeks) (mean ± SEM)		29.1 ± 0.6
Gestational age at delivery (days) (mean ± SEM)	39.9 ± 0.1	31.9 ± 0.4
Interval from PROM to delivery (days) (mean ± SEM)		19 ± 3
First serum sample (weeks) (mean ± SEM)	29.9 ± 0.4	30.7 ± 0.4
Cesarean section (%)	26	31
Birth weight of the newborn (g) (mean ± SEM)	3657 ± 97	1846 ± 85
Apgar scores < 7 at 1 min	0	28
Use of corticosteroids (%)	0	84
Use of antibiotics (%)	0	97

2.2 Study protocols and laboratory analyses

Study IV

In Study IV, the examinations involved questionnaires, clinical investigations, physical performance measurements and laboratory investigations (Nissinen *et al.* 1993). Briefly, body weight was measured in light clothing to the nearest 100 g. Two measurements of blood pressure were performed by a trained nurse, on the right arms of men in a sitting position after five minutes rest. The mean of two measurements was used.

Glucose tolerance was tested using a 75 g oral glucose load as described by Stengård *et al.* (1993). The participants were asked to fast for at least 12 hours and the tests were carried out between 08.00 and 12.00 a.m. Blood glucose was measured in venous plasma using the glucose dehydrogenase method. Insulin analyses after fasting and 2 hours after the glucose load were performed using Pharmacia Diagnostica Phadeseph Insulin RIA kits.

Total and high-density lipoprotein (HDL) cholesterol concentrations were analyzed in fresh sera by an enzymatic method using an Olli C 3000 photometer (Kone Ltd., Espoo, Finland). Levels of HDL-cholesterol were measured after precipitation of very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) cholesterol by the dextran-magnesium chloride method (Kostner 1976), and serum triglycerides were assayed after enzymatic hydrolyzation and determination of the liberated glycerol by colorimetry, with commercial reagents, using a KONE C automatic discrete analyzer.

Study V

Serum C-reactive protein (CRP) concentrations were measured by immunoturbidimetry (Harmoinen *et al.* 1981). Subclinical intrauterine infection was defined as a serum CRP concentration exceeding 12 mg/L (Kurki *et al.* 1990).

2.3 Definitions of glucose tolerance and metabolic syndrome (IV)

Glucose intolerance included both impaired fasting glucose (IFG) and impaired glucose tolerance (IGT). Diabetes, IFG and IGT were all defined according to the 1999 WHO definition (Alberti *et al.* 1998). In IFG, the venous plasma fasting glucose level was 5.6–6.1 mmol/L and the two-hour post-glucose load was < 7.8 mmol/L. In IGT, the venous plasma fasting glucose level was under 6.1 mmol/L and the two-hour post-glucose load was 7.8–11.0 mmol/L. In diabetes, the venous plasma fasting glucose level was ≥ 7.0 mmol/L and/or the two-hour post-glucose load was ≥ 11.1 mmol/L, or clinical diagnosis of diabetes was assessed on the basis of dietary, oral, or insulin treatment.

Metabolic syndrome was defined according to the European Group for the Study of Insulin Resistance (Balkau *et al.* 1999). Hypertension was defined according to the definition agreed by the International Society of Hypertension, and the Sixth Joint National Committee

recommendations (Joint National Committee on prevention, detection and treatment of high blood pressure 1997). Thus, metabolic syndrome was defined as hyperinsulinemia, fasting hyperglycemia or diabetes (fasting serum glucose > 6.1 mmol/L) and at least two of the following: obesity (BMI ≥ 30 kg/m^2), dyslipidemia (serum triglycerides ≥ 1.7 mmol/L and/or serum HDL cholesterol < 0.9 mmol/L), or hypertension (blood pressure ≥ 140/90 or use of blood pressure medication). Hyperinsulinemia was estimated on the basis of fasting insulin levels in the uppermost quartile of the nondiabetic population.

2.4 Mortality data (IV)

Mortality data were systemically collected from the Finnish Death Register. Death certificates and hospital records were collected for all the men who died between 1989 and 1997. A single reviewer recorded the cause of death in order to minimize variability. In 1997, the vital status of all subjects was ascertained through the Finnish Population Registry. In addition, for the study population, all hospital discharge diagnoses, with the ninth revision of the International Classification of Disease (IDC-9) (WHO 1977) codes 410–414, 426–438 and 440–448 were identified from the National Hospital Discharge Register, and hospital records were collected and reviewed. The person coding the causes of death was blind to the risk factor status of the subject. Where multiple causes of death were recorded, priority was given to accidents, advance-stage cancer, coronary heart disease and stroke. Total, cardiovascular and CHD mortality were used as outcome parameters.

2.5 Statistical methods

Study IV

Statistical analyses were performed using SPSS for Windows version 11.0. Pearson's correlation coefficients were calculated for IGFBP-l, SHBG, testosterone and TSH levels, plus a number of cardiovascular risk factors and components of metabolic syndrome. Age- and BMI-adjusted mean values of IGFBP-1 and SHBG levels in metabolic syndrome, glucose intolerance and diabetes were estimated using general linear models. Relative risks (with 95% confidence intervals) of death from all causes, cardiovascular causes and coronary heart disease were estimated using Cox regression analyses.

Study V

Differences between mean values were compared by analysis of variance and paired-samples *t* tests. Spearman's correlation coefficients were calculated to examine bivariate relationships. A value of $p < 0.05$ was considered statistically significant. The results were presented as mean ± SEM.

RESULTS

1. *IN VITRO* STUDIES

1.1 Insulin and IGFs in the production of IGFBP-1 and SHBG (I)

Over 90% of the cells were viable at the end of the experiments, and no difference in viability between control and experimental wells was found. At 6 h no changes were observed in IGFBP-1 or SHBG production.

At a 30 nM concentration, insulin attenuated both IGFBP-1 and SHBG production by about 35% from the control value at 48 h. At a 30 nM concentration, IGF-I inhibited IGFBP-1 and SHBG production by 20 and 24 percent, respectively. At a 30 nM concentration, IGF-II inhibited IGFBP-1 production by 32%, but for SHBG the decrease caused by IGF-II was only 11%. (Table 5)

Table 5. Regulation of IGFBP-1 and SHBG production by insulin, IGFs and estradiol (I, II)

	IGFBP-1	SHBG
Insulin	↓	↓
IGF-I	↓	↓
IGF-II	↓	↓
estradiol	ns	↑

ns: no significant changes

At a 30 nM concentration, IGF-I caused a 40% decrease in IGFBP-1 RNA levels, whereas insulin and IGF-II reduced them by 52%. The changes in SHBG RNA levels brought about by all hormones were smaller than those in IGFBP-1 RNA levels, 10–25 percent, and only the IGF-I-induced change was statistically significant

1.2 Estradiol in the production of IGFBP-1 and SHBG (II)

The release of IGFBP-1 from HepG2 cells was 209.6 ± 3.5 (SEM) ng/μg DNA during the 48-h experiment, showing no significant increase in the presence of estradiol (E2). Likewise, the intracellular IGFBP-1 concentration showed no significant change (14.6 ± 0.2 and 15.7 ± 0.5 ng/μg DNA) in the presence of E2 at concentrations of 10 nmol/L to 2.5 μmol/L (Table 5).

Unlike the absence of IGFBP-1 responses to E2, a positive SHBG response to E2 was observed in HepG2 cells. At an E2 concentration of 0.5 μmol/L, the intracellular SHBG concentration increased from 9.8 ± 0.2 to 17.3 ± 0.5 pg/μg DNA ($p < 0.0001$), and at 2.5 μmol E2/L it increased from 9.8 ± 0.2 to 19.4 ± 0.5 pg/μg DNA ($p < 0.0001$). However, no increased release of SHBG into culture medium was found (Table 5).

The amount of DNA was similar in all wells, including the controls. The variation was between 27.58 and 32.90 μg/well in all groups. Cell growth was thus not affected by the amounts of E2 added.

1.3 Regulation of IGFBP-3 by various hormones (III)

Insulin stimulated IGFBP-3 release from HepG2 cells at concentrations of 1 nmol/L and higher. This increase was 2-fold at an insulin concentration of 30 nmol/L. Like insulin, IGF-I and IGF-II also enhanced IGFBP-3 secretion. The maximal increases (2-fold for IGF-I and 2.5-fold for IGF-II) were observed with IGF concentrations of 30 nmol/L. (Table 6) The results concerning intracellular concentrations were similar. Total RNA was increased 1.7- to 2-fold.

Table 6. Regulation of IGFBP-3 production by various hormones (study III)

	IGFBP3
Insulin	↑
IGF-I	↑
IGF-II	↑
estradiol	ns
tamoxifen	ns
testosterone	ns
cortisol	↓

ns: no significant changes

Estradiol increased IGFBP-3 release at a supraphysiological concentration of 2.5 μmol/L, but not at lower concentrations of 10–500 nmol/L. Testosterone and tamoxifen had no effect on IGFBP-3 production at concentrations up to 2.5 μmol/L. Cortisol inhibited IGFBP-3 secretion at physiological concentrations. None of the effectors altered the total protein concentrations in the experimental wells. At the end of the experiment, cell viability was over 90 percent in test and control wells.

2. CLINICAL STUDIES

2.1 IGFBP-1 and SHBG in metabolic syndrome and cardiovascular risk (IV)

In age-adjusted analysis, men with metabolic syndrome, glucose intolerance or diabetes had significantly lower mean values of SHBG concentrations compared with the rest of the men. The association between low SHBG levels and glucose intolerance and diabetes also remained significant after adjusting for BMI. For IGFBP-1 there was a significant inverse association with metabolic syndrome and glucose intolerance in age-adjusted analysis, but neither of the associations remained significant after adjusting for BMI.

Fasting serum SHBG and IGFBP-1 concentrations showed several correlations with various cardiovascular risk factors in Pearson correlation analyses (Table 7). Levels of SHBG showed more frequent and stronger inverse correlations than IGFBP-1. Concentrations of SHBG correlated with fasting and two-hour glucose levels in the oral glucose tolerance tests,

whereas IGFBP-1 levels showed no correlation. Concentrations of both SHBG and IGFBP-1 correlated with fasting and two-hour insulin levels in OGTTs.

Table 7. Pearson correlation coefficients for CVD risk factors and studied factors

	SHBG	IGFBP-1	testosterone	TSH
Age	-0.027	0.120 *	-0.110 *	-0.108 *
BMI	-0.228 **	-0.258 **	-0.258 **	0.015
Total cholesterol	0.017	0.036	0.055	0.015
HDL cholesterol	0.328 **	0.132 *	0.266 **	-0.103
Triglycerides	-0.318 **	-0.140 *	-0.226 **	0.120 *
Fasting glucose	-0.266 **	-0.011	-0.224 **	-0.024
2 h post-OGTT glucose	-0.211 **	-0.106	-0.114	0.063
Fasting insulin	-0.383 **	-0.287 **	-0.275 **	0.148 *
2 h post-OGTT insulin	-0.295 **	-0.217 **	-0.190 **	0.157 *
Systolic blood pressure	-0.127 *	0.058	-0.008	0.034
Diastolic blood pressure	-0.101	0.019	0.009	0.027

BMI: body mass index, OGTT:oral glucose tolerance test, CVD: cardiovascular disease
**P<0.01 *P<0.05

In Cox regression analysis, the lowest quartile of SHBG concentrations was related to an increased risk of cardiovascular and CHD mortality, whereas the lowest quartile of IGFBP-1 levels was not related to an excess risk of either cardiovascular or CHD mortality. The highest quartile values of SHBG and IGFBP-1 showed no correlations with increased mortality.

Testosterone concentrations showed correlations with cardiovascular risk factors similar to those of SHBG, but they did not correlate with systolic blood pressure. Plasma testosterone levels had a strong inverse association with glucose intolerance, metabolic syndrome and diabetes.

The concentrations of thyroid hormones showed correlations with cardiovascular risk factors. Levels of TSH had no association with glucose intolerance or diabetes, but were associated with metabolic syndrome.

2.2 IGFBP-3 and IGF-I in PROM and preterm delivery (V)

In the women with PROM, serum IGF-I and IGFBP-3 levels were not statistically different from those in the control subjects. Seventeen women with PROM showed elevated CRP levels (35 ± 6 [SEM] mg/L). In this group, the levels of serum IGF-I and IGFBP-3 did not differ from those in the control subjects, or in the 15 PROM women with normal CRP levels. Serial serum samples before delivery were obtained from 22 women with PROM. No consistent changes in IGF-I or IGFBP-3 concentrations were seen during the follow-up period of 9 ± 2 days. The last antepartum levels of IGF-I and IGFBP-3 correlated positively with the birth weight of the newborns in the PROM group ($r = 0.417$, $p = 0.018$ for IGF-I; $r = 0.483$, $p = 0.005$ for IGFBP-3) but not in the control group ($r = 0.069$, $p = 0.733$ for IGF-I; $r = -0.223$, $p = 0.264$ for IGFBP-3).

DISCUSSION

1. *IN VITRO* STUDIES

1.1 Cell cultures

Culture of HepG2 cells is widely used as an experimental model for studies on hepatic IGFBP and SHBG production. HepG2 cells are highly differentiated human hepatoblastoma cells, which exhibit most of the characteristics of normal hepatocytes (Knowles *et al.* 1980). They are easier to obtain and culture than normal hepatocytes and grow in a more uniform manner. Naturally, the results of cell culture studies have limitations and the results should be viewed together with those from studies carried out *in vivo*. Whether normal liver cells respond to exogenous stimuli in the same way as HepG2 cells is not certain. Culture conditions might affect the results. As HepG2 cells are often grown on in a laboratory for a long time, subclones with characteristics different from those in the original cell line might occur. This can in some cases explain different results from different laboratories. The cells used in this study were obtained directly from the American type culture collection and were divided as few times as possible.

1.2 Regulation of IGFBP-1 and SHBG by insulin and IGFs

The results of insulin infusion studies have previously shown that insulin regulates IGFBP-1 levels (Suikkari *et al.* 1988, 1989b), with a nonlinear inverse correlation between portal vein insulin concentrations and serum IGFBP-1 levels (Conover *et al.* 1992). As IGFBP-1 values are downregulated 18-fold more than SHBG levels in insulin clamp studies (Ebeling *et al.* 1995), the initial hypothesis in this study was that the effect of insulin on hepatic IGFBP-1 production might be greater than on SHBG production. However, no such difference was observed in the present *in vitro* study using hepatoma cells, as the decrease in production was found to be similar for both proteins.

Insulin-like growth factor-I was found to decrease SHBG and IGFBP-1 production in HepG2 cells to a lesser extent than insulin. The result is in concordance with that in a previous study

(Lee *et al.* 1993) in which reduction of IGFBP-1 production by IGF-I was observed. However, this has not been observed in all studies (Singh *et al.* 1990). The changes in SHBG concentrations were similar to those in previous investigations on HepG2 cells (Plymate *et al.* 1988, Crave *et al.* 1995). Like insulin, IGF-I downregulated IGFBP-1 and SHBG in the same manner. In clinical studies on patients treated with either IGF-I or growth hormone, which increases IGF-I levels, an inverse correlation between plasma insulin and SHBG levels has been found, but no correlation between IGF-I and SHBG levels (Gafny *et al.* 1994). Likewise, in postmenopausal breast cancer patients, multivariate analysis has revealed that plasma SHBG concentrations correlate inversely with those of insulin, but not with those of IGF-I (Lønning *et al.* 1995). Serum IGFBP-1 and IGF-I levels have also been shown to correlate inversely (Hall *et al.* 1988, Wang *et al.* 1989, Lønning *et al.* 1995).

Information on the effects of IGF-II on IGFBP-1 and SHBG synthesis is scarce. In the present study, IGF-II, like insulin and IGF- I, decreased production of both IGFBP-1 and SHBG. This effect was, however, smaller for SHBG than for IGFBP-1. The biological significance of this finding remains unresolved.

The reduced IGFBP-1 release from HepG2 cells was probably a result of reduced synthesis, because IGF-I, IGF-II and insulin all downregulated IGFBP-1 RNA levels. This confirms the results of other studies (Powell *et al.* 1991, Lee *et al.* 1993). SHBG RNA was significantly reduced by IGF-I only. Reduction of SHBG mRNA by insulin has been shown in a previous study in which the incubation time was much longer than in this study (Loukovaara *et al.* 1995).

On the basis of the present *in vitro* data, it is concluded that insulin, IGF-I and IGF-II decrease both IGFBP-1 and SHBG protein production with similar maximal responses. The findings suggest that both IGFBP-1 and SHBG concentrations could act as highly sensitive indicators of insulin bioactivity, and thus be possible markers of abnormal glucose tolerance and metabolic syndrome. This hypothesis was tested in the clinical study (IV).

1.3 Regulation of IGFBP-1 and SHBG by estrogen

Insulin is the major regulator of IGFBP-1 and SHBG secretion, as has been shown both *in vivo* and *in vitro* in many studies (Conover *et al.* 1990, Singh *et al.* 1990, Mercier-Bodard *et al.* 1991, Powell *et al.* 1991, Plymate *et al.* 1988). The thyroid hormones thyroxine and tri-iodothyronine (Mercier-Bodard *et al.* 1989, Angervo *et al.* 1993a, 1993b), as well as the IGFs (Plymate *et al.* 1988, 1990, Lee *et al.* 1993) have been shown to regulate the two binding proteins in a similar fashion. However, there are clinical situations in which the changes in IGFBP-1 levels cannot be explained by changes in the concentrations of insulin or thyroid hormones. For instance, controlled ovarian hyperstimulation is accompanied by rising serum IGFBP-1 levels in the follicular phase, correlating with rising E2 levels, both in serum and in follicular fluid (Seppälä *et al.* 1984, 1988, Martikainen *et al.* 1992). The most likely sources of this IGFBP-1 are the liver and/or the ovary, because the third potential source, the endometrium, does not produce IGFBP-1 during the estrogen-dominated proliferative phase of the cycle (Koistinen *et al.* 1993).

This study addressed the issue of whether the high serum IGFBP-1 concentrations observed during ovarian stimulation are of hepatic or nonhepatic origin, possibly resulting from stimulation of secretion from ovarian cells. In normal conditions, most of the circulating IGFBP-1 appears to be of hepatic origin. Thus it is logical to assume that the changes caused by estrogens would reflect the effects of estrogen on hepatic IGFBP-1 production. However, during ovarian stimulation, IGFBP-1 production by ovarian granulosa cells is greatly increased and this might be the reason for the observed high IGFBP-1 concentrations.

Serum IGFBP-1 and SHBG concentrations correlate with each other in many situations (Lønning *et al.* 1995), and SHBG has been found to be responsive to estrogens *in vitro* (Loukovaara *et al.* 1995) and *in vivo* (Odlind *et al.* 1982). The effect of estrogens is likely to be indirect, as there is no estrogen-responsive element in the SHBG gene (Hammond *et al.* 1987). The data on transcriptional regulation is controversial (Mercier-Bodard *et al.* 1991, Crave *et al.* 1995, Loukovaara *et al.* 1995). In this study, increased intracellular SHBG concentrations in response to estradiol showed that the cells had retained their responsiveness under the culture conditions employed. Therefore, the absence of changes in IGFBP-1 concentrations indicates that estradiol does not affect IGFBP-1 synthesis in these hepatoma

cells, as also confirmed in other studies (Mercier-Bodard *et al.* 1991). However, it is possible that some characteristics of HepG2 subclones may differ.

The present results indicate that estradiol has little effect on hepatic IGFBP-1 secretion provided that normal liver cells respond to exogenous stimuli in the same way as HepG2 cells. Given that the ovarian compartment contributes to the circulating IGFBP-1 pool (Seppälä *et al.* 1988, Martikainen *et al.* 1992), the results of the present study support the clinical hypothesis that the major source contributing to the circulating IGFBP-1 pool, the liver, does not appear to contribute significantly to the elevated IGFBP-1 levels observed in response to the rising estrogen levels during controlled ovarian hyperstimulation.

1.4 Regulation of IGFBP-3 by insulin, IGFs, sex hormones and cortisol

In the present study, insulin stimulated IGFBP-3 secretion from HepG2 cells even at concentrations that correspond to postprandial insulin levels in portal blood. The rapid effect suggests that insulin may regulate hepatic IGFBP-3 production under physiological conditions. Furthermore, the results may partly explain why IGFBP-3 levels are low in diabetic patients before insulin treatment is started (Bereket *et al.* 1995). The high sensitivity of HepG2 cells to insulin suggests that insulin effects are likely to be mediated directly through its own receptors, or via stimulation of IGF-I production. IGF-I and IGF-II also enhanced IGFBP-3 production from HepG2 cells. These results in liver carcinoma cells are in accordance with clinical observations that show that administration of IGF-I to patients with non-insulin-dependent diabetes mellitus increases serum IGFBP-3 levels (Lieberman 1992). Our results are also in accord with those of previous *in vitro* studies carried out with different types of hepatoma cells (Gucev *et al.* 1997, Scharf *et al.* 1998).

The release of IGFBP-3 was not influenced by sex steroid hormones at physiological concentrations. This is in keeping with the results of clinical studies showing no fluctuation of IGFBP-3 levels during the menstrual cycle (Thierry van Dessel *et al.* 1996) and the fact that there is no gender difference in circulating IGFBP-3 concentrations after puberty (Juul *et al.* 1995). High supraphysiological estradiol concentrations resulted in a slightly greater release of IGFBP-3, but this effect was small compared with SHBG concentrations in the same experiments. There is some discrepancy between the results of *in vivo* studies and the present *in vitro* study regarding the effects of glucocorticoids on IGFBP-3 levels. *In vivo*,

dexamethasone increases IGFBP-3 serum concentrations in healthy males (Miell *et al.* 1993), whereas we found a decrease *in vitro*. The limitations of cell culture studies could account for this, but it is also possible that *in vivo* confounding factors can affect the results.

It is concluded that insulin and both IGFs stimulate IGFBP-3 production by cultured human hepatoma cells. In contrast, sex steroids and tamoxifen have no effect on IGFBP-3 secretion, whereas cortisol inhibits it.

2. CLINICAL STUDIES

2.1 Study populations

In study IV the study population consisted of elderly men aged 70–89. As in all studies on elderly people, survivor bias may influence the findings, as the subjects most sensitive to cardiovascular risk factors may be already ill or dead. The absence of any significant difference in the number of deaths between different glucose tolerance categories may reflect this, as diabetes, abnormal glucose tolerance and metabolic syndrome should be important risk factors as regards death. The reason for this observation may also be that, unlike in proportional hazard analysis, time is not accounted for. The high prevalence of CVD in the study group indicates that there were still enough risk-sensitive subjects alive.

This study addressed only men and similar studies on women or both genders have not yet been performed. In women, high testosterone values are connected with increased cardiovascular risk and metabolic syndrome (Apridonidze *et al.* 2004) and the effect of estrogen is stronger than in men, so the results could be different.

2.2 IGFBP-1 and SHBG in metabolic syndrome, diabetes and mortality

In the present study we compared, in the same cohort, SHBG and IGFBP-1 as surrogate markers of glucose intolerance, metabolic syndrome, diabetes, cardiovascular risk and cardiovascular mortality.

Individuals at an increased risk of type 2 diabetes, such as those with impaired glucose tolerance, identified via OGTTs, greatly need to be identified, as they form an important target group for diabetes prevention measures. Prevention of type 2 diabetes has been shown to be feasible. In prevention studies with goals of weight reduction, dietary changes and exercise, the overall incidence of diabetes was reduced by 58 percent (Tuomilehto *et al.* 2001, Knowler *et al.* 2002). Diabetes criteria have been updated at regular intervals, with the most recent one being given by the American Diabetes Association (ADA), where the oral glucose tolerance test is no longer recommended. However, using the fasting glucose criterion alone, subjects with isolated post-challenge hyperglycemia, consisting of one-sixth of diabetic patients, will remain undiagnosed (The DECODE Study Group 1999).

As expected from their common regulators, such as insulin and thyroid hormones, both SHBG and IGFBP-1 concentrations were found to correlate inversely with glucose intolerance and metabolic syndrome in the same fashion. However, low SHBG concentrations were also associated with prevalent diabetes while low IGFBP-1 levels were not. Moreover, the association between SHBG levels and diabetes was less influenced by adjustment for BMI, suggesting that the effect of SHBG on glycemia was independent of obesity. However, because of the known association between SHBG and androgens, the present results on men point to a need for further studies on both genders, with a broader range of age. In a recent study of 20- to 39-year-old women with PCO, low SHBG concentrations were associated with metabolic syndrome (Apridonidze *et al.* 2004).

Fasting hyperinsulinemia has been shown to increase the risk of fatal CVD (Hu *et al.* 2004). However, in one study concerning elderly men without diabetes, hyperinsulinemia alone did not appear to increase the risk (Oh *et al.* 2002). Older men with isolated post-challenge hyperglycemia have an increased mortality rate similar to that in other diabetic patients (The DECODE Study Group 1999). These patients cannot be identified without an OGTT, which is time-consuming and therefore not suitable for screening. As the basal SHBG level correlates

with two-hour glucose and insulin in the OGTT, it might provide useful information in those cases in which post-challenge hyperglycemia cannot be determined.

This study is the first to show an association between coronary heart disease mortality and low SHBG levels in men, even though the association between low SHBG concentrations and abnormal glucose tolerance, metabolic syndrome or diabetes, conditions known to increase the risk of cardiovascular disease (Lakka *et al.* 2000), has also been observed in previous prospective studies (Goodman-Gruen and Barrett-Connor 1996, Haffner *et al.* 1996). The main difference between our study and a previous study showing no association between SHBG levels and CVD deaths (Goodman-Gruen and Barrett-Connor 1996) is that our study group was older and had more prevalent diseases at baseline. After adjustment for abnormal glucose tolerance the association between low SHBG levels and CVD or CHD mortality remained significant, indicating that glucose tolerance does not explain this association. Prevalent CVD is the strongest predictor of CVD mortality. In the present study the association between low SHBG levels and CHD or CHD mortality was no longer significant after adjustment for prevalent CVD. This is logical, because our study population consisted of elderly men and the prevalence of CVD was high at the outset. Nevertheless, SHBG levels did not differ between men with and without prevalent CVD at baseline.

Low IGFBP-1 concentrations did not have the same association with CHD as low SHBG levels, so we were not able to confirm the findings of a recent investigation in which it was concluded that low baseline IGFBP-1 levels increase the risk of fatal ischemic heart disease among elderly predominantly nondiabetic men and women without prevalent cardiovascular disease (Laughlin *et al.* 2004). In our study group there were more diabetics and subjects with prevalent disease and the follow-up time was longer. The association between low IGFBP-1 levels and an unfavorable cardiovascular risk profile (Harrela *et al.* 2000) is concordant with the results of this study. The unexplained association between high IGFBP-1 levels and mortality observed in the same population as ours at a younger age (Harrela *et al.* 2002) was no longer evident, possibly because the sensitivity of the aging population to risk factors is likely to change.

The reasons for discordance between low SHBG and low IGFBP-1 levels with respect to cardiovascular and CHD death, despite their similar association with cardiovascular risk factors, are unclear. As insulin is the major regulator of both of these binding proteins, it is

possible that glucose metabolism does not explain the observed discordance. As sex hormones are also important regulators of SHBG, they may play a role here.

Insulin increases ovarian androgen secretion in women (Nestler *et al.* 1997), whereas in men the association between testosterone and insulin concentrations is inverse, independent of age (Simon *et al.* 1992). The observed strong associations of low SHBG and low testosterone levels with abnormal glucose tolerance, metabolic syndrome and diabetes are concordant with somewhat higher insulin levels, as also observed by other investigators (Björntorp 1991, Simon *et al.* 1992). Prospective studies have also shown that low levels of SHBG and testosterone may predict the development of type 2 diabetes (Haffner *et al.* 1996). Whether the relationship between low testosterone levels and abnormal glucose metabolism is direct or indirect is not known, as the relationship between testosterone and insulin is complex (Wu *et al.* 2003). Previous studies, however, show no consistent association between testosterone concentrations and coronary heart disease or mortality (Muller *et al.* 2003, Wu *et al.* 2003).

That both low SHBG and low testosterone concentrations were connected with abnormal glucose tolerance and cardiovascular risk factors in this study is somewhat surprising, as SHBG levels usually rise with age, whereas those of testosterone tend to fall. However, similar findings have been reported by other investigators (Goodman-Gruen and Barrett-Connor 1996, Laughlin *et al.* 2004). In elderly men, total testosterone but not free bioavailable testosterone has been associated with the development of type 2 diabetes (Oh *et al.* 2002). The effect of androgen therapy could be either beneficial or harmful, as both high and low testosterone levels have been associated with a higher risk (Lindstedt *et al.* 1991) and, in animal studies, high-dose testosterone has been reported to worsen insulin resistance (Holmäng *et al.* 1992). In relatively small trials, androgen treatment has improved insulin sensitivity in middle-aged abdominally obese men (Marin *et al.* 1992, 1995), but the findings have not been consistent (Liu *et al.* 2003). The results of some studies suggest that exogenous administration of androgenic steroids to men or women leads to insulin resistance (Landon *et al.* 1962, Cohen *et al.* 1987). In other studies, administration of testosterone has improved insulin sensitivity, probably through improvements in body composition and a reduction in the levels of circulating nonesterified fatty acids (Marin *et al.* 1992, 1995). A window for optimal insulin sensitivity has been suggested, as both elevated and low testosterone concentrations can reduce body sensitivity to insulin (Björntorp 1991). The results of most studies suggest a less atherogenic lipid profile with increasing endogenous total testosterone

(Barrett-Connor 1995). Testosterone could be an independent risk factor for cardiovascular diseases or its effects may be mediated via other risk factors such as insulin levels.

In conclusion, as initially hypothesized, low serum SHBG and IGFBP-1 concentrations may both indicate abnormal glucose tolerance and metabolic syndrome. Circulating levels of IGFBP-1 and SHBG also correlate with other known risk factors and surrogate markers of cardiovascular risk. Whereas IGFBP-1 levels show no association with cardiovascular morbidity or mortality, low SHBG concentrations are associated with increased cardiovascular mortality.

2.3 IGFBP-3 and IGF-I in PROM and preterm delivery

As both pregnancy and infection influence IGFBP-3 concentrations and protease activity (Langford *et al.* 1995, de Martino *et al.* 2000), and IGF-I levels have been found to decrease before delivery (Wang *et al.* 1991), it can be hypothesized that IGFBP-3 concentrations could be altered in PROM, chorioamnionitis and subsequent preterm birth.

In term and preterm deliveries, many endocrinological changes have been found to be similar. For instance, the cascade of events leading to preterm birth represents a normal signal with inappropriate timing rather than an abnormal signal (Steer *et al.* 1990). This also applies to IGF-I and IGFBP-1, the serum levels of which are similar during term and preterm parturition (Wang *et al.* 1993).

A number of changes occur in the IGF system during pregnancy. For instance, serum IGF-I (Wilson *et al.* 1982) and IGFBP-3 levels (Langford *et al.* 1995) rise with advancing gestational age. To our knowledge no data are available on these levels in women with PROM and preterm delivery, before regular uterine contractions appear. PROM invariably leads to preterm delivery (Cox *et al.* 1988), as also found in this study. We found that IGF-I and IGFBP-3 levels in women with PROM were similar to those in normal pregnancy, suggesting absence of their involvement in the pathogenesis of PROM or the pretem delivery that follows. We cannot exclude the possibility that antibiotics and/or corticosteroids given to women with PROM may affect serum IGF-I or IGFBP-3 levels.

Premature rupture of membranes is associated with chorioamnionitis in up to 70% of cases (Naeye *et al.* 1980). Therefore CRP concentrations were measured routinely to detect emerging intrauterine infection. Interestingly, the elevation of CRP levels had no association with the levels of either IGF-I or IGFBP-3. The reason may be that the emerging infection was local, because in generalized infection serum IGF-I and IGFBP-3 levels are low, as shown in children with perinatal human immunodeficiency virus infection and serious disease (de Martino *et al.* 2000). Low IGFBP-3 levels have also been found in other severely ill patients (Davies *et al.* 1991) and after surgery (Davenport *et al.* 1992). Therefore, IGF-I and IGFBP-3 may not serve as indicators of an acute phase reaction in chorioamnionitis.

It is concluded that serum IGF-I and IGFBP-3 levels in women with PROM and preterm delivery, with and without intrauterine infection, do not differ from those in controls. This suggests that maternal serum IGF-I and IGFBP-3 do not appear to play any major role in preterm birth, and the changes in the IGF system are similar to those in normal birth.

CONCLUSIONS

On the basis of the present work, the following conclusions can be drawn:

1. The downregulation of IGFBP-1 and SHBG production caused by insulin and IGFs in hepatoma cells was found to be similar. This could indicate their similar ability to serve as possible markers of cardiovascular risk in insulin-resistant states.

2. Estradiol stimulation resulted in no changes in hepatic cell IGFBP-1 secretion, while SHBG secretion was significantly increased. These results suggest that the liver does not appear to contribute significantly to the elevated IGFBP-1 levels seen in response to high estrogen levels during ovarian stimulation.

3. Insulin, IGF-I and IGF-II stimulated IGFBP-3 production in HepG2 cells. Sex hormones or the antiestrogen tamoxifen did not influence IGFBP-3 release at physiological concentrations. Cortisol had an inhibitory effect on IGFBP-3 production.

4. Subjects with metabolic syndrome or abnormal glucose tolerance had low serum SHBG and IGFBP-1 concentrations. Only SHBG (low level) was also associated with diabetes. The association between SHBG and glycemia was less dependent on obesity, vs. IGFBP-1. Both SHBG and IGFBP-1 levels also correlate with other known cardiovascular risk factors. Low SHBG levels were related to CVD mortality, whereas low IGFBP-1 levels were not.

5. Serum IGF-I and IGFBP-3 levels remained unaltered in women with PROM. This suggests that maternal serum IGF-I and IGFBP-3 do not play any major role in preterm birth.

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